ORIGINAL ARTICLE

A C G publications

*J.Chem.Metrol.*14:2 (2020) 142-152

journal of chemical metrology

Development of TLC method for simultaneous estimation of novel combination of amlodipine besylate, rosuvastatin calcium, and fimasartan potassium in synthetic mixture

Devansh Kansara[1*], Usmangani K. Chhalotiya[1*]

Hetaben M. Kachhiya[1] and Ishita Patel[1]

[1]*Department of Pharmaceutical Chemistry and Analysis, Indukaka Ipcowala College of Pharmacy, Beyond GIDC, P.B. No. 53, Vitthal Udyognagar- 388 121, Gujarat, India*

(Received July 23, 2020; Revised September 13, 2020; Accepted September 17, 2020)

Abstract: An accurate, sensitive, robust and precise high performance thin layer liquid chromatography method was developed based on ICH Q2 (R1) guidelines for estimation of novel combination of Amlodipine besylate, Rosuvastatin calcium and Fimasartan potassium in bulk and its synthetic mixture. Pre-coated silica gel aluminum plate 60 F_{254} was selected as the stationary phase and *n*-hexane, *n*-butanol, methanol, and Glacial Acetic Acid (5.7:2:2.3:0.1, v/v/v/v) was selected as mobile phase. All three drugs showing appreciable absorbance at the common wavelength of 242 nm were selected for quantification of Amlodipine besylate, Rosuvastatin calcium, and Fimasartan potassium, respectively. The method was validated for linearity, precision, accuracy, and robustness, limit of detection and limit of quantitation as per ICH parameters. The regression coefficients (r^2) were found to be 0.9986, 0.9975 and 0.9988 for Amlodipine besylate, Rosuvastatin calcium, and Fimasartan potassium, respectively. The average percentage recovery of Amlodipine besylate, Rosuvastatin calcium and Fimasartan potassium were found to be 99.38-100-60%, 99.75-100.63%, 99.39-100%, respectively. Thin Layer Chromatographic method has prospective qualitative as well as quantitative applications for concurrent estimation of Amlodipine besylate, Rosuvastatin calcium and Fimasartan potassium in bulk and pharmaceutical dosage form.

Keywords: HPTLC; amlodipine besylate (AML); rosuvastatin calcium (ROS); fimasartan potassium (FIM); validation.

1. Introduction

According to WHO (World Health Organization), hypertension is causes death of approx. 7.5 million which is about 12.8% of total of all death per annum. There are different stages of high blood pressure like pre-hypertension, mild hypertension, moderate hypertension and severe hypertension [1].

Amlodipine besylate, Rosuvastatin calcium, and Fimasartan potassium are used to decrease high blood pressure. Chemically, Amlodipine besylate (AML) is 3-ethyl-5-methyl-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate and having an empirical formula of $C_{26}H_{31}ClN_2O_8S$ with a molecular weight of 567.05 g/mol.

* Corresponding author: E-Mail: usmangani84@gmail.com

The article was published by ACG Publications
http://www.acgpubs.org/journal/journal-of-chemical-metrology © July-December 2020 EISSN:1307-6183
DOI:http://doi.org/10.25135/jcm.45.20.07.1744

Mechanism of action is angio-selective calcium channel blocker which results in inhibition of the contraction of cardiac muscles [2].

Chemically, Rosuvastatin calcium (ROS) is (3R, 5S, 6E)-7-(4-(4-fluorophenyl)-6-(1-methylethyl)-2-(ethyl(methylsulfonyl)amino)-5-pyrimidinyl)-3,5-dihydroxy-6-heptenoic acid with an empirical formula of $C_{22}H_{28}FN_3O_6S$ and having a molecular weight 1001.14 g/mol. The mechanism of action of ROS is an inhibitor of enzyme HMG-CoA reductase which is a rate-limiting enzyme for conversation of the 3-hydroxy-3-methylglutarate to mevalonate which is the starting compound for cholesterol synthesis, ROS, along with a good and balanced diet, helps to reduce harmful LDL and helps to conserve the HDL [3].

Chemically, Fimasartan potassium (FIM) is 2-(2-butyl-4-methyl-6-oxo-1-{[2'-(1H-1,2,3,4 tetrazol-5-yl)-[1,1'-biphenyl]-4-yl]methyl}-1,6-dihydropyrimidin-5-yl)-N,N dimethylethanethioamide with an empirical formula of $C_{27}H_{36}KN_7O_4S$ and having a molecular weight 593.79 g/mol. It is an angiotensin II receptor antagonist and recommended for the treatment of hypertension and cardiac failure [4]. Double combination of these drugs have been used before but current novel triple combination of AML, ROS, and FIM is under phase III clinical trials and the study has been completed in month of June 2019 as per USFDA notification [5]. The chemical structures of all three active pharmaceutical ingredients are shown in Figure 1.

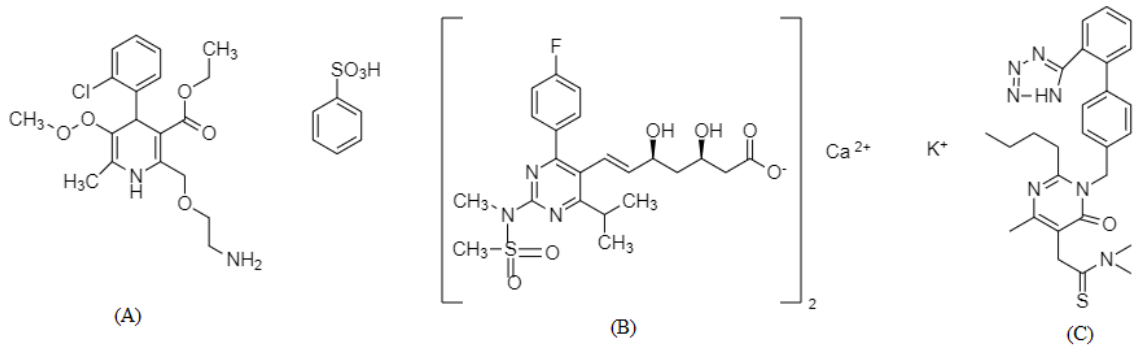

Figure 1. Structures of (**A**) Amlodipine besylate, (**B**) Rosuvastatin calcium and (**C**) Fimasartan potassium.

From a detailed literature review, we found that various liquid chromatographic methods [6-19] and HPTLC [20-22] methods have been published for the estimation of AML, ROS, and FIM in individual, combination with the any two of above active pharmaceutical ingredients and combination with other drugs. Till date no HPTLC method has been published so far for proposed triple combination. The advantages of HPTLC method is to analyze large number of samples with less amount of mobile phase, time and cost of analysis require less and also it is much faster and eco-friendly as compared to LC. The present work describes the sensitive, accurate, precise, and robust TLC method developed for the qualitative and quantitative analysis of proposed novel triple combination and method was validated as per the ICH guideline Q2(R1).

2. Experimental

2.1. Chromatographic Condition

For sample introduction, Hamilton of 100µL sample syringe was used with the help of Camag Linomat 5 (CAMAG, Switzerland) sample applicator on pre-coated silica gel aluminum plate 60 F_{254} was used which is having 10 cm x 10 cm with 0.2 mm thickness, supplied by E. Merck, Germany. Camag TLC scanner was used for the densitometric scanning with HPTLC system controlled using Camag winCATS software, Version 1.4.8. For weight of all the three drugs and excipients on calibrated Sartorius CP124S (Sartorius Corporation, United States) was used.

2.2. Reagents and Material

AML, ROS, and FIM are obtained from well-known pharmaceutical industry, Gujarat, India. Different solvents like HPLC grade of methanol, AR grade of *n*-Hexane and *n*-Butanol are procured from SRL Diagnostic Pvt. Ltd. Mumbai and Astron chemicals Pvt. Ltd., India. Microcrystalline cellulose and Calcium phosphate are procured from Chiti Chem Corporation Pvt. Ltd., Vadodara, India, Cross povidone, Magnesium stearate, Hydroxy propyl methyl cellulose K100M, and Cross carmellose Sodium are obtained from Chemdyes Corporation Pvt. Ltd, Vadodara, India. Whatman filter paper 42 was procured from Merck KGaA, Germany.

2.3. Preparation of Synthetic Mixture [23]

Synthetic mixture of the triple combination of drug with excipients was prepared for 20 tablets in the following manner. Accurately weighed excipients like Microcrystalline cellulose (4080 mg), Cross Povidone (300 mg), Calcium Phosphate (300 mg), Magnesium Stearate (111 mg), HPMC (Hydroxy propyl methyl cellulose) K100M (30 mg), Cross Carmellose Sodium (150 mg) were transferred into a mortar with pestle and mixed well. Into the above mortar pestle accurately weighed 200 mg of Amlodipine besylate, 400 mg of Rosuvastatin calcium, and 1200 mg of Fimasartan potassium were added and mixed well.

From the above mixture, accurately weight equivalent to 10 mg of AML, 20 mg of ROS, and 60 mg of FIM were transferred into a 100 mL volumetric flask containing 50.0 mL methanol, sonicated for 15 minutes to remove dissolved gases and improve the solubility of active pharmaceutical ingredients and the volume was made up to the mark with the same solvent. The solution was then filtered with the help of Whatman filter paper no. 42 in another 100 mL of volumetric flask and the volume was adjusted up to the mark with methanol to obtain the final concentration of AML 100 µg/mL, 200 µg/mL for ROS, and 600 µg/mL for FIM, respectively.

2.4. Preparation of Stock Solution

The concentrations of1000 µg/mL for AML, 2000 µg/mL for ROS, and 6000 µg/mL for FIM were prepared individually by dissolving accurately weighed 10 mg of AML, 20 mg of ROS, and 60 mg of FIM in three different 10 mL of volumetric flask, swirl to dissolve and volume were adjusted upto the mark with methanol for each drug. Pipetted out 1.0 mL of aliquot from each of above stock solutions of AML, ROS, and FIM respectively were taken into 10 ml volumetric flask and diluted upto mark with methanol to obtain working standard solution of 100 µg/mL of AML, 200 µg/mL of ROS, and 600 µg/mL of FIM, respectively.

2.5. Preparation of Stock Solution

A narrow bands having 8 mm length with 9 mm distance between two bands of standard and sample solutions of AML, ROS, and FIM were applied on Aluminium pre-coated TLC plate.

2.6. Mobile Phase Preparation

Solvents mixture selected as a mobile phase, n-Hexane: *n*-Butanol: Methanol: Glacial Acetic Acid (5.7:2:2.3:0.1, v/v/v/v) were measured individually, mixed well and added in a twin-trough glass chamber and kept for chamber saturation for 44 min with the mobile phase vapors at 25 ± 2 °C and managed with air conditioning system.. Ascending development method was used for development and was allowed to migrate a distance of 70 mm. After that, TLC plates were removed and dried with a hair dryer.

2.7. Densitometric Analysis

Using winCATS planar chromatography 1.4.8 software, densitometric scanning was performed in the absorbance mode. The bands were scanned at wavelength 242 nm with a scanning rate of 20 mm/s. By using a linear regression equation, concentrations of the compound were chromatographed and the intensity of diffusely reflected light is determined and evaluated as peak areas against concentrations (ng/band).

2.8. Validation

As per International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guidelines Q2 (R1) for the validation parameters, validation of the developed TLC method was carried out [24].

For linearity and range, calibration curves at seven different concentrations over a range of 200-1400 ng/band for AML, 400-2800 ng/band for ROS, and 600-8400 ng/band for FIM were carried out. With the help of the winCATS 1.4.8 software, calibration was performed for five times and calibration curves were developed by plotting peak area versus concentration (n=5).

LOD and LOQ is nothing but sensitivity of proposed method. By using linear regression model, these parameters were calculated from the slope and standard deviation of the intercept of calibration curves. By scanning a blank for six times, noise can be determined. LOD was calculated as 3 times of the noise level and LOQ was calculated 10 times of the noise level

The standard stock solution of 100 µg/mL of AML, 200 µg/mL of ROS, and 600 µg/mL of FIM were used for determination of precision. Intraday precision was calculated by analyzing solution of AML, ROS, and FIM at three different levels covering of lower, medium and higher concentration of the calibration curve three times on the same day. Interday precision was estimated by analyzing sample solution of AML, ROS, and FIM at three levels covering lower, medium and higher concentration period of three days. The peak areas were used to calculate mean percentage (%) RSD value.

Repeatability precision was evaluated in terms of injection and scanning repeatability. The standard stock solution of 100 µg/mL of AML, 200 µg/mL of ROS, and 600 µg/mL of FIM were prepared and used for the repeatability study. Repeatability of the injection was studied by applying six time of middle concentration of calibration range of 800 ng/band for AML, 1600 ng/band for ROS, and 4800 ng/band for FIM. Scanning repeatability was studied by scanning the band of middle concentration of calibration range for six times.

As per ICH guidelines, the accuracy was performed by calculating % recovery of AML, ROS and FIM by standard spiking method at three different levels 80, 100, and 120 %. Known amount of standard AML (480 ng/band, 600 ng/band, and 720 ng/band), standard ROS (960 ng/band, 1200 ng/band, and 1440 ng/band) and standard FIM (2880 ng/band, 3600 ng/band, and 4320 ng/band) were taken from standard stock solution of 100 µg/mL of AML, 200 µg/mL of ROS, and of 600 µg/mL of FIM. It was added to pre quantified sample and the amount of AML, ROS, and FIM were estimated by measuring the peak area by fitting these value to the straight line equation of calibration curve.

For robustness study, small deliberate changes in different chromatographic conditions like the chamber saturation time, temperatures, wavelength, and mobile phase composition of the proposed developed method were selected at concentration level of 800 ng/band for AML, 1600 ng/band for ROS, and 4800 ng/band for FIM, respectively. The mean R_f values and %RSD were calculated.

The specificity of method was checked by analyzing AML, ROS, and FIM in the presence of different excipients used in synthetic mixture. By comparing R_f values and densitogram of synthetic mixture with standards, the chromatographic peaks of AML, ROS, and FIM were confirmed

2.9. Analysis of Synthetic Mixture

For the preparation of synthetic mixture, AML, ROS, and FIM was taken in the ratio of 10: 20: 60 mg, respectively. Common excipients like Microcrystalline cellulose (4080 mg), Cross Povidone (300 mg), Calcium Phosphate (300 mg), Magnesium Stearate (111 mg), (HPMC) Hydroxypropyl methylcellulose K100 (30 mg), Cross Carmellose Sodium (150 mg) were weighed accurately and transfer into mortar pestle along with 200 mg of AML, 400 mg of ROS, and 1200 mg of FIM which is equivalent to preparing 20 tablets. Weight accurately equivalent to 10 mg of AML, 20 mg of ROS, and 60 mg of FIM transfer it a into 10 mL volumetric flask containing 5.0 mL of methanol and sonicated for 15 min for improving solubility of drugs and removing dissolved gases. By using Whatman filter paper No.42, the solution was filtered and collects the filtrate in another 10 mL volumetric flask and the residue remaining on filter paper was washed with few mL of methanol, both filtrate and residue washing are combined in above 10 mL volumetric flask and volume was adjusted up to the mark with the methanol. Form the above volumetric flask, Pipetted out 1.0 mL aliquot, transferred it in another 10 mL volumetric flask and volume was adjusted up to the mark with methanol to obtained final strength 100 µg/mL for AML, 200 µg/mL for ROS, and 600 µg/mL for FIM, respectively.

3. Results and discussion

3.1. Optimization of Mobile Phase

By trial and error method, the selection of the mobile phase was carried out. Different composition solvents were tried for the optimizations mobile phase like Methanol: Toluene, Methanol: Acetonitrile, Toluene: n-Butanol, *n*-Hexane: Ethyl acetate, *n*-Hexane: n-Butanol in different proportions. On the basis of above trials the mobile phase, *n*-Hexane: *n*-Butanol: Methanol: Glacial Acetic Acid (GAA) (5.7:2:2.3:0.1, v/v/v/v) showed good peak symmetry with well resolved peaks of AML, ROS and FIM, respectively (Figure S5, see supporting information). Chamber saturation time was 44 minutes and diffusion of the analyte bands occurs because of solvent migration distances greater than 70 mm. Therefore, to validate chromatographic conditions, well resolved with good symmetry of peaks were obtained by the use of mobile phase *n*-Hexane: *n*-Butanol: Methanol: GAA (5.7:2:2.3:0.1, v/v/v/v) with a chamber saturation time of 44 minutes at 25 ºC and solvent migration distance is 70 mm.

3.2. Method Validation

Linearity relationship over the concentration range was found to be 200-1400 ng/band for AML having regression coefficient of 0.9988, concentration ranges from 400-2800 ng/band for ROS with regression coefficient of 0.9977 and concentration ranges from 1200-8400 ng/band for FIM with regression coefficient of 0.9988. Figure 2 shows a 3D overlay of HPTLC densitogram of the calibration concentration ranges of AML, ROS, and FIM at 242 nm. The statistical data of calibration curve is shown in Table 1.

Table 1. Statistical data of calibration curve

Parameter	AML	ROS	FIM
Conc.(ng/band)	200-1400	400-2800	1200-8400
Regression co-efficient (r^2)	0.9986	0.9978	0.9988
Slope of regression equation	2.9	6.56	1.51
Standard deviation of slope	0.06	0.24	0.01
Intercept of regression	111.5	10260	3013
Standard deviation of intercept	40.23	348.72	44.28
Detection limit (ng/band)	42.80	151.75	283.70
Quantification limit (ng/band)	129.71	459.88	859.70

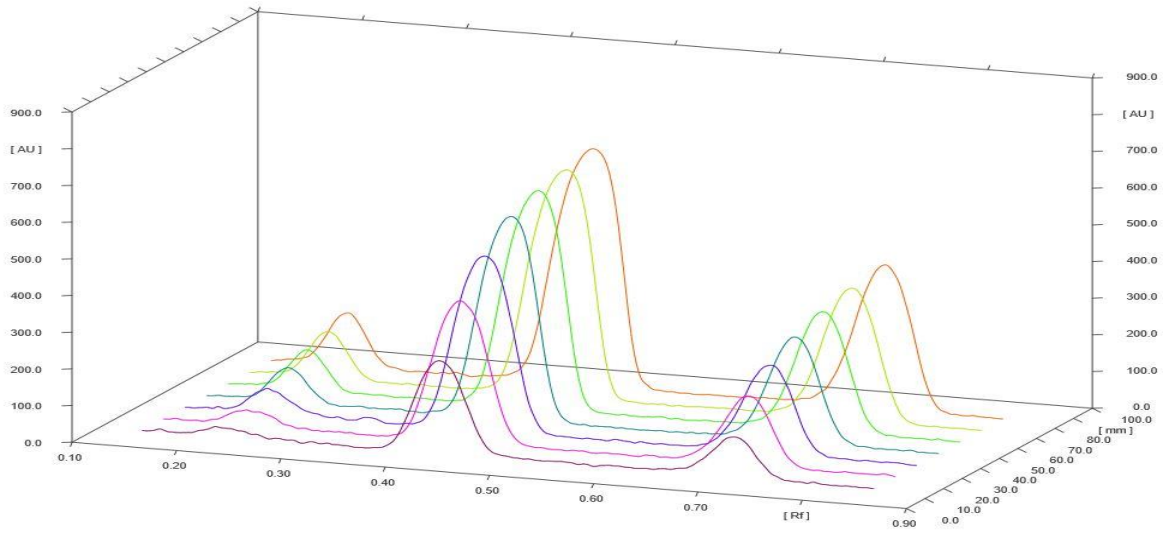

Figure 2. Three dimensional overlay densitogram of Amlodipine besylate (200-1400 ng/band), Rosuvastatin calcium (400-2800 ng/band) and Fimasartan potassium (600-8400 ng/band) using *n*-Hexane: *n*-Butanol: Methanol: Glacial Acetic Acid (5.7: 2: 2.3:0.1ml v/v/v/v) at 242 nm.

From the Intraday and interday precision study data, % RSD was found to be less than 3.0% which represent the proposed method is precise. From the scanning and injection repeatability data, the %RSD was found to be less than 3.0% which represent the proposed method is repeatable.

The accuracy study of proposed method was carried out by calculating percentage (%) recovery of drug from synthetic mixture by standard spiking method at three levels 80%, 100%, and 120% as per ICH guidelines. For AML(480 ng/band, 600 ng/band, and 720 ng/band) were spiked, for ROS (960 ng/band, 1200 ng/band, and 1440 ng/band) were spiked and for FIM (2880 ng/band, 3600 ng/band, and 4320 ng/band) were spiked in pre-quantified sample solution which were prepared from synthetic mixture. From the data, the percentage (%) recovery of 99.38-100.63% for AML, 99.75-100.63% for ROS, and 99.39-100% for FIM was obtained. The result of proposed accuracy studies is shown in Table 2.

LOD was found to be 42.8 ng/band for AML, 151.75 ng/band for ROS and 283.7 for FIM respectively, LOQ was found to be 129.71 ng/band for AML, 460 ng/band for ROS and 860 ng/band for FIM, respectively. The peak purity from the densitogram was found to be more than 0.999 which compiles the purity of peak and peak purity spectra of calibration range is shown in Figure S6 (see supporting information).

Robustness was performed for selected chromatographic conditions and data were evaluated based on peak R*f* value of the band. The result of proposed robustness studies is shown in Table 3.

For solution stability, the solutions of sample and standard of AML, ROS and FIM were evaluated at ambient temperature (25 ± 2 °C).. The test was carried out over a time period of 2, 4, 8 and 24 hours of time period and the percentage (%) amount of drug was found to be >98% by the developed HPTLC method which indicate drugs were found to be stable for cited period of time. Table 4 data are representing the summary of validation parameters of proposed method.

Table 2. Result of proposed accuracy studies

Drug	% Level of spiking	Amount of drug taken from mixture (ng/band)	Amount of standard drug spiked (ng/band)	Mean Area ± SD (n=3)	Amount of drug recovered (ng/band)	Recovery %
AML	0	600	0	1840.53±31.41	596.33	99.38
	80	600	480	3248.1±56.11	601.82	100.30
	100	600	600	3585.7±82.76	598.27	99.71
	120	600	720	3949.11± 69.31	603.61	100.63
ROS	0	1200	0	18184.52±191.85	1207.6	100.63
	80	1200	960	24415.5± 76.24	1197.07	99.75
	100	1200	1200	26004.67± 92.57	1199.23	99.93
	120	1200	1440	27577.67±273.03	1198.92	99.91
FIM	0	3600	0	8451.67± 115.51	3600.23	100
	80	3600	2880	12768.33±63.10	3578.16	99.39
	100	3600	3600	138587.7± 84.1	3580.05	99.46
	120	3600	4320	14951.57± 104.18	3583.67	99.54

Table 3. Robustness study of proposed analytical method

Parameters	**Normal Condition**	**Change in condition**	**Mean R*f* value ± SD (n=3)**			**RSD %**		
			AML (800 ng/ band)	ROS (1600 ng/ band)	FIM (4800 ng/ band)	AML (800 ng/ band)	ROS (1600 ng/ band)	FIM (4800 ng/ band)
Change in Mobile Phase Ratio	*n*-Hexane: *n*-Butanol: Methanol: Glacial Acetic Acid (5.7:2:2.3:0.1, v/v/v/v)	(5.8:1.9:2.3:0 .1, v/v/v/v)	0.21± 0.005	0.43±0.01	0.70± 0.02	2.7	2.32	2.94
		(5.6:2.1:2.3:0 .1, v/v/v/v)	0.22±0.005	0.43±0.01	0.70± 0.01	2.71	2.64	2.16
Chamber saturation	44 min	42 min	0.20± 0.005	0.44± 0.01	0.71± 0.01	2.79	2.27	1.40
		46 min	0.21± 0.005	0.44±0.005	0.72± 0.01	2.64	1.30	1.38
Change in wavelength	242 nm	244 nm	0.20± 0.002	0.43±0.003	0.71± 0.001	2.93	2.36	2.65
		240 nm	0.21 ±	21622.79± 515.52	10341.25± 289.71	1.82	2.38	2.80
Change in temperature of working area	25 ºC	20 ºC	0.21±0.002	0.44±0.003	0.71± 0.005	2.73	2.44	2.21
		30 ºC	0.21±0.004	0.43±0.004	0.70± 0.005	1.8	2.16	2.70

Table 4. Summary of validation parameters of proposed analytical methods

Parameters	AML	ROS	FIM
Linearity (ng/band)	200-1400	400-2800	1200-8400
Retention factor	0.21	0.42	0.7
Detection limit (ng/band)	42.80	151.7	283.70
Quantitation limit (ng/band)	129.71	459.87	859.7
Accuracy (%)	99.38-100.63	99.75-100.63	99.39-100
Precision (%RSD)			
Intra-day (n=3)	1.01-1.63	0.72-1.88	0.96-1.57
Inter-day (n=3)	0.7-2.25	2.64-2.84	2.07-2.34
Specificity	Specific	Specific	Specific
Robustness	Robust	Robust	Robust
Solution stability	Stable for 24 h	Stable for 24 h	Stable for 24 h

3.3. Analysis of synthetic mixture

The proposed HPTLC method is successfully applied for the determination of AML, ROS, and FIM in their synthetic mixture. The percentage (%) amount of drug for was found to be more than 98% AML, ROS, and FIM, respectively. The chromatogram of standard and marketed formulation of AML (800 ng/band), ROS (1600 ng/band), and FIM (4800 ng/band) has been carried out. Figure S7 (see supporting information) is representing the densitogram of synthetic mixture.

4. Conclusion

By use of proposed HPTLC method, identification and quantification of Amlodipine besylate, Rosuvastatin calcium and Fimasartan potassium has been carried out from bulk and synthetic mixture. As per ICH guidelines, the proposed validated method is found to be selective, sensitive, accurate, precise, robust, less time consuming, cost effective and eco-friendly as compared to LC technique [21].

Acknowledgements

All authors are very thankful to Indukaka Ipcowala College of Pharmacy, New Vallabh Vidyanagar and SICART, Vallabh Vidyanagar for provided that with necessary facilities to carry out research work.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/journal-of-chemical-metrology

ORCID
Devansh Kansara: 0000-0001-7377-8663
Usmangani K. Chhalotiya: 0000-0003-1183-2761
Hetaben M. Kacchiya: 0000-0003-4534-6243
Ishita Patel: 0000-0002-0021-5576

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